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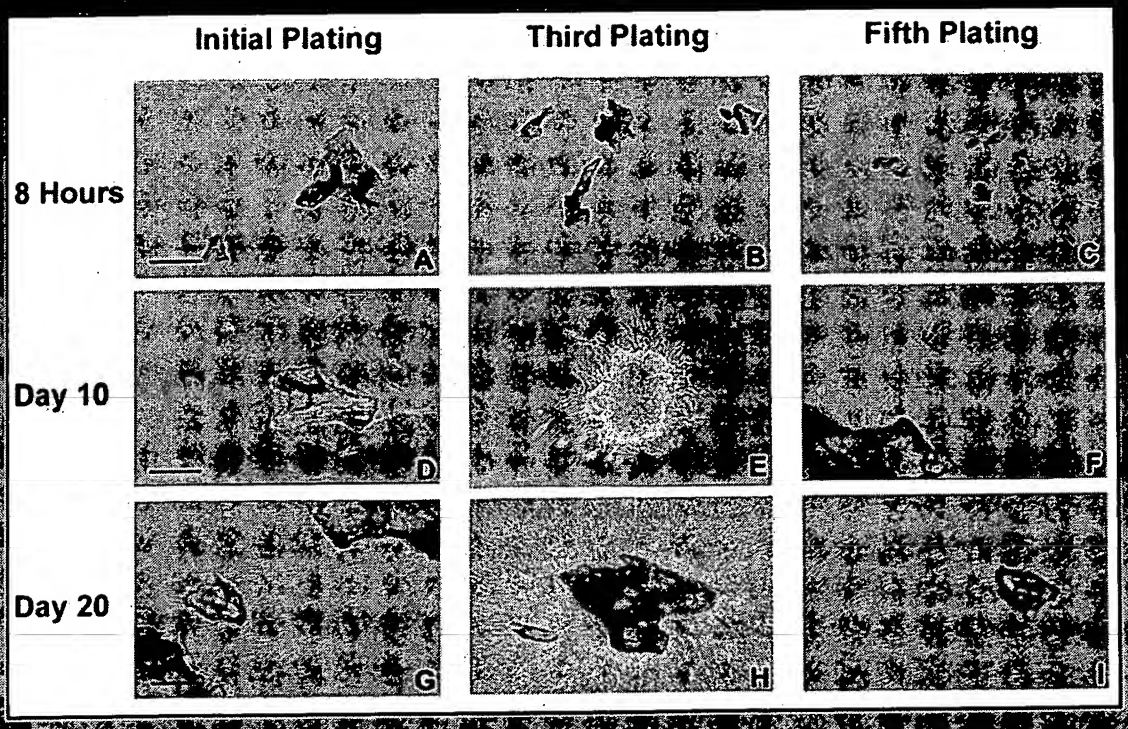

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A Simple, High-Yield Method for Obtaining Multipotential Mesenchymal Progenitor Cells from Trabecular Bone

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Abstract

In vitro cultures of primary, human trabecular bone-derived cells represent a useful system for investigation of the biology of osteoblasts. Our recent discovery of the multilineage mesenchymal differentiation potential of trabecular bone-derived cells suggests the potential application of these cells as mesenchymal progenitors for tissue repair and regeneration. Such applications are crucially dependent on efficient cell-isolation protocols to yield cells that optimally proliferate and differentiate. In this study, we describe a simple, high-yield procedure, requiring minimal culture expansion, for the isolation of mesenchymal progenitor cells from human trabecular bone. Moreover, these cells retain their ability to differentiate along multiple mesenchymal lineages through successive subculturing. Cell populations isolated and cultured as described here allow the efficient acquisition of a clinically significant number of cells, which may be used as the cell source for tissue-engineering applications.

Index Entries: Mesenchymal progenitor cell; human trabecular bone; tissue engineering; isolation procedure; in vitro cell culture.

1. Introduction

The in vitro culture of human trabecular bone-derived cells has served as a useful system for the investigation of the biology of osteoblasts. These cells reproducibly exhibit several characteristics of the osteoblast phenotype, in particular the ability to form a mineralized extracellular matrix (ECM) composed primarily of collagen type I, and the expression of high levels of alkaline phosphatase and osteocalcin, which can be further stimulated by the secosteroid 1,25-dihydroxyvitamin D3 ($1,25[OH]_2D_3$) *(1–6)*. Additionally, these trabecular bone-derived cells are capable of producing all the major noncollagenous proteins of the bone ECM *(7,8)*. More recently, in vitro cultures of these cells have also provided insights into the interactions between bone and biomaterial for orthopedic and dental implants and into the response of osteoblasts to different growth factors and hormones, cell–matrix interactions, and osteoblast differentiation and maturation *(9–12)*.

The recent discovery in our laboratory of the multilineage mesenchymal differentiation potential of cells derived from collagenase-treated human trabecular bone fragments has prompted further interest, in view of the potential application of these cells as mesenchymal progenitors in the repair and regeneration of tissue damaged by disease or trauma *(13)*. However, such applications require a clinically significant number of cells of the appropriate phenotype. Although serial subculture of primary cells may be used to generate sufficient numbers of cells, loss of phenotype may occur when diploid cells are cultured for extended periods *(14)*, and the choice of a particular medium for promoting the viability and proliferation of particular cell type is often lacking in precision and

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prior experience *(15,16)*. Although a sufficient number of cells may be generated through the use of transformed, immortalized cell lines, the genetic component of such cells is often compromised in unknown ways.

In this study we introduced a simple, high-yield procedure that allows the isolation and production of a significant number of mesenchymal progenitor cells from human trabecular bone with a minimal need for culture expansion. Moreover, these cells retain their ability to differentiate along multiple mesenchymal lineages through successive subculturing, making them appropriate candidate cells for a broad range of tissue-engineering applications.

2. Materials and Methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated, and were of highest grade and purity.

2.2. Isolation of Human Trabecular Bone-Derived Cells

Normal trabecular bone specimens were obtained from the femoral heads of patients (female 79 yr old, female 63 yr old, male 46 yr old) undergoing total hip arthroplasty, and were processed under sterile conditions within a laminar-flow hood. The femoral head was washed repeatedly at the exposed surface, aspirated of the adherent marrow, wrapped in sterile gauze, and placed tightly within a C-clamp device, leaving the trabecular surface completely exposed. A 36-mm acetabular reamer and base (Zimmer, Warsaw, IN) attached to a 14.4-V drill (Black & Decker, Hampstead, MD) was used to grind the trabecular bone out of the femoral head. After sufficient bone was obtained, the chips were vigorously washed with sterile 0.9% NaCl (Baxter Laboratories, Deerfield, IL) and agitated repeatedly. For comparison purposes, trabecular bone from the same patient was also processed as described previously by Robey and Termine *(8)* and modified by Sinha and Tuan *(13)* and Noth et al. *(17)*. Briefly, the trabecular bone of the femoral head was curetted, extensively minced with straight microdissecting scissors, and washed. The resulting bone chips from each isolation protocol were then treated in the same way. Following the washings, bone fragments were incubated in Dulbecco's modified Eadle's medium (DMEM)/F-12K medium (Specialty Media, Phillipsburg, NJ) supplemented with 50 µg/mL ascorbate, 256 U/mL collagenase type XI, 2 m*M* L-glutamine, and 50 µg/mL penicillin–streptomycin, and transferred to a spinner flask in a humidified incubator at 37°C with 5% CO_2 for 3–4 h. Following digestion of cellular material, the bone fragments were extensively washed again with sterile 0.9% NaCl and plated into 150-cm^2 polystyrene cell-culture flasks (Corning Inc., Corning, NY) in calcium-free DMEM/F-12K medium supplemented with 10% fetal bovine serum (FBS) (Premium Select; Atlanta Biologicals, Atlanta, GA), 50 µg/mL ascorbate, 2 m*M* L-glutamine, and 50 µg/mL penicillin–streptomycin with changes of medium every 3–4 days. Once 70–80% confluence was reached, cells were removed with 0.25% trypsin containing 1 m*M* ethylenediamine tetraacetic acid (EDTA) (Gibco BRL, Life Technologies, Grand Island, NY) and utilized for study.

2.3. Replating of Trabecular Bone Fragments

Processed trabecular bone fragments were plated in low-calcium DMEM/F-12K medium and kept in culture until the migrating cells had grown to 70–80% confluence. After the cells were removed from the tissue culture plate with trypsin, the same bone fragments were extensively washed and agitated with sterile 0.9% NaCl, and were replated into new tissue culture dishes. In determining the cell yield per gram of trabecular bone, the fragments were maintained in culture for 4 wk increments before being replated.

2.4. Differentiation of Cell Cultures Along Mesenchymal Lineages

Adipogenesis of confluent monolayer cultures was induced using DMEM (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 1 µ*M* dexamethasone, 0.5 m*M* 3-isobutyl-1-methylxanthine (IBMX), 1 µg/mL insulin, 100 µ*M* indomethacin, and 50 µg/mL

penicillin-streptomycin *(18)*. Cultures were maintained for 2 wk with medium changes every 3–4 days. Osteogenic induction of confluent monolayer cultures was accomplished using DMEM/F-12K supplemented with 10% FBS, 50 µg/mL ascorbate, 10 m*M* β-glycerophosphate, and 0.1 µ*M* dexamethasone for 21 d with medium changes every 3–4 days. Control cultures were maintained without adipogenic and osteogenic supplements, respectively. Chondrogenesis of high-density pellet cultures (2×10^5 cells/pellet, 500*g* for 5 min) was induced using serum-free DMEM supplemented with 50 µg/mL ascorbate, 0.1 µ*M* dexamethasone, 40 µg/mL L-proline, 100 µg/mL sodium pyruvate, and ITS-plus (Collaborative Biomedical Products, Cambridge, MA) *(18–21)*. Recombinant human transforming growth factor-β1 (TGF-β1; R&D, Minneapolis, MN) was also added at a final concentration of 10 ng/mL. Pellet cultures were maintained for 21 d with changes of medium every 3–4 days. Control pellet cultures were maintained without the addition of TGF-β1. Monolayer cultures of cells maintained in the low-calcium, serum-containing medium, which were left untreated, were designated as undifferentiated.

2.6. Reverse Transcription Polymerase Chain Reaction Analysis

Total cellular RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For efficient yield, chondrogenic pellet cultures were first briefly homogenized in Trizol Reagent using a pestle (Kontes, Vineland, NJ). RNA samples were reverse-transcribed using random hexamers and the SuperScript First Strand Synthesis System (Gibco BRL, Life Technologies, Grand Island, NY). Amplification of complementary DNA (cDNA) with the polymerase chain reaction (PCR) was done with AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT) and the gene-specific primer sets listed in **Table 1**. Thirty-two cycles of amplification were used for all genes, and consisted of 1 min of denaturation at 95°C, 1 min of annealing at 57°C (for the genes for collagen II, IX, XI, aggrecan, Sox 9) or 51°C (for all remaining genes), and 1 min of polymerization at 72°C, followed by a final 10-min extension at 72°C. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a control for RNA loading of samples. PCR products were analyzed electrophoretically on an ethidium bromide 2% MetaPhor agarose gel (BioWhittaker, Rockland, ME).

2.7. Alkaline Phosphatase Histochemical Analysis

Osteogenic monolayer cultures were stained histochemically for alkaline phosphatase (Sigma, cat. no. 86-C) according to the manufacturer's protocol.

2.8. Cell-Proliferation Assay

Undifferentiated cells were added to a 96-well plate (1×10^3 cells/well) and assessed for cell viability and proliferation at d 1, 2, 4, 8, 16, and 24 with the CellTiter 96 AQ_{ueous} One Solution MTS Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a colored formazan product was measured spectrophotometrically on the basis of absorbance at 490 nm (A_{490}).

2.9. Statistical Analysis

Data presented represent the combined results of three separate culture experiments established from three total hip arthroplasty procedures. In each experiment, comparisons were made from cultures established from the same patient. Student's *t* test was performed to determine the statistical significance ($p \leq 0.05$) of the differences observed between cultures derived from the curetted bone fragments and those from the reamed bone fragments.

3. Results

3.1. Comparison of Cells Isolated Through The Reaming and Curetting Methods

3.1.1. Cell Morphology

Phase-contrast microscopy of trabecular bone fragments isolated from the same patient through the two different bone-explant techniques used in

Table 1
RT-PCR Primers for Differentiation-Specific Gene Expression Analysis: Sequence and Expected Product Size

Gene		Primer Sequences (5'-3')	Position (bp)	Expected Product Size (bp)
Housekeeping gene				
GAPDH	Sense:	GGGCTGCTTTTAACTCTGGT	134–835	702
	Antisense:	TGGCAGGTTTTTCTAGACGG		
Bone-specific genes				
ALP	Sense:	TGGAGCTTCAGAAGCTCAACACCA	122–575	454
	Antisense:	ATCTCGTTGTCTGAGTACCAGTCC		
BSP	Sense:	AATGAAAACGAAGAAAGCGAAG	549–998	450
	Antisense:	ATCATAGCCATCGTAGCCTTGT		
Col IA2	Sense:	GGACACAATGGATTGCAAGG	3209–3669	461
	Antisense:	TAACCACTGCTCCACTCTGG		
OC	Sense:	ATGAGAGCCCTCACACTCCTC	19–312	294
	Antisense:	GCCGTAGAAGCGCCGATAGGC		
Adipose-specific genes				
LPL	Sense:	GAGATTTCTCTGTATGGCACC	1261–1536	276
	Antisense:	CTGCAAATGAGACACTTTCTC		
PPARγ2	Sense:	GCTGTTATGGGTGAAACTCTG	153–503	351
	Antisense:	ATAAGGTGGAGATGCAGGCTC		
Cartilage-specific genes				
Col II	Sense:	CAGGTCAAGATGGTC	1341–1717	377
	Antisense:	TTCAGCACCTGTCTCACCA		
Col IX	Sense:	GAAAATGAAGACCTGCTGG	126–641	516
	Antisense:	GAAAAGGCTGCTGTTTGGAGAC		
Aggrecan	Sense:	TGAGGAGGGCTGGAACAAGTACC	6591–6910	350
	Antisense:	GGAGGTGGTAATTGCAGGGAACA		
Sox 9	Sense:	ATCTGAAGAAGGAGAGCGAG	548–811	264
	Antisense:	TCAGAAGTCTCCAGAGCTTG		

Abbreviations: ALP, alkaline phosphate; BSP, bovine sialoprotein; Col IA2, collagen type 1 alpha 2; OC, osteocalcin; LPL, lipoprotein lipase; PPARγ2, peroxisome proliferator activated receptor-gamma 2; Col II, collage type II; Col IX, collagen type 9.

the study revealed that smaller, thinner, more jagged chips were obtained by reaming of the femoral head (**Fig. 1A,B**). Furthermore, cell proliferation from the two populations of bone fragments proceeded at different rates (**Fig. 1C–F**). Ten days after culture was established in the low-calcium DMEM/F-12K medium, cells obtained by the curetting method (**Fig. 1C**) had just begun to migrate from the fragments, whereas cells were rapidly migrating and proliferating from the reamed fragments (**Fig. 1D**). Microscopic observations after 20 d revealed a more dramatic difference with cells derived from the reamed fragments approaching confluence (**Fig. 1E**), whereas cells from the curetted chips were sparse and remained low in number. Cell morphology (**Fig. 1D,E**) was similar for the two populations, revealing homogeneous, fibroblast-shaped cells.

3.1.2. Cell Yields Derived from Replated Bone Fragments

After 4 wk, we determined the yields of cells derived from primary cultures of curetted and reamed bone fragments (**Fig. 2A,B**). Similar to the data presented in **Fig. 1**, cells derived from the reamed-bone-explant cultures approached 80% confluence, totaling $2.150 \times 10^6 \pm 0.265 \times 10^6$ cells/g of wet bone fragments, whereas curetted fragments generated $1.75 \times 10^6 \pm 0.066 \times 10^6$ cells/g after the first 4 wk in culture. Upon successive sub-

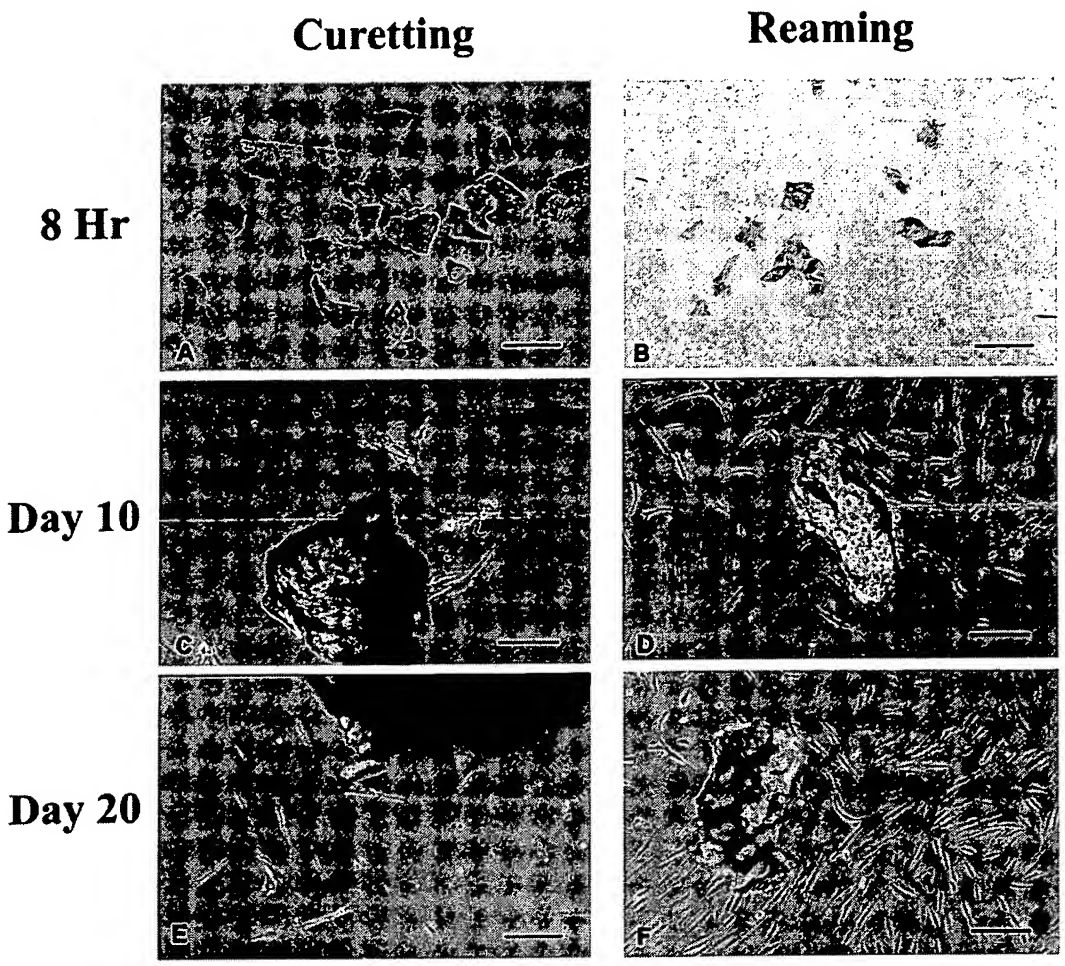


Fig. 1. Morphology of trabecular explant cultures processed by curetting (**A**,**C**,**E**) and reaming (**B**,**D**,**F**) of the femoral head. (**A**,**B**) Appearance of the reamed trabecular bone fragments after plating revealed smaller, thinner chips with larger surface areas than curetted fragments (bar = 0.4 mm). (**C**,**D**) After 10 d in culture, cells began to migrate and proliferate more rapidly from the reamed bone chips (bar = 0.1 mm). (**E**,**F**) Dramatic differences in cell number and colony formation became more apparent after 20 d in culture (bar = 0.1 mm).

culture of the bone fragments, a significant decrease in cell production was seen for the curetted fragments (0.678×10^6 cells/g) in the second plating, and by the third plating, cellular output completely tapered off, with 0.068×10^6 cells/g after an additional 4 wk of culture. On the other hand, cell yields per gram of reamed trabecular-bone fragments were consistently maintained through four consecutive platings at 4-wk intervals, diminishing slightly to 1.477×10^6 cells/g after the fifth plating. The total number of cells generated per gram of reamed bone ($9.68 \times 10^6 \pm 0.324 \times 10^6$) after five successive platings of the bone chips was more than 3-fold greater than that generated from curetted chips ($2.93 \times 10^6 \pm 0.189 \times 10^6$ cells/g).

3.1.3. Analysis of Osteogenic Gene Expression

Confluent monolayer cultures of cells derived from fragments isolated by the two bone-explant procedures used in the study served as controls for the analysis of osteogenic gene expression (**Fig. 3**). Although these control cultures expressed messenger RNA (mRNA) for collagen type I at similar levels, other osteogenesis-specific marker

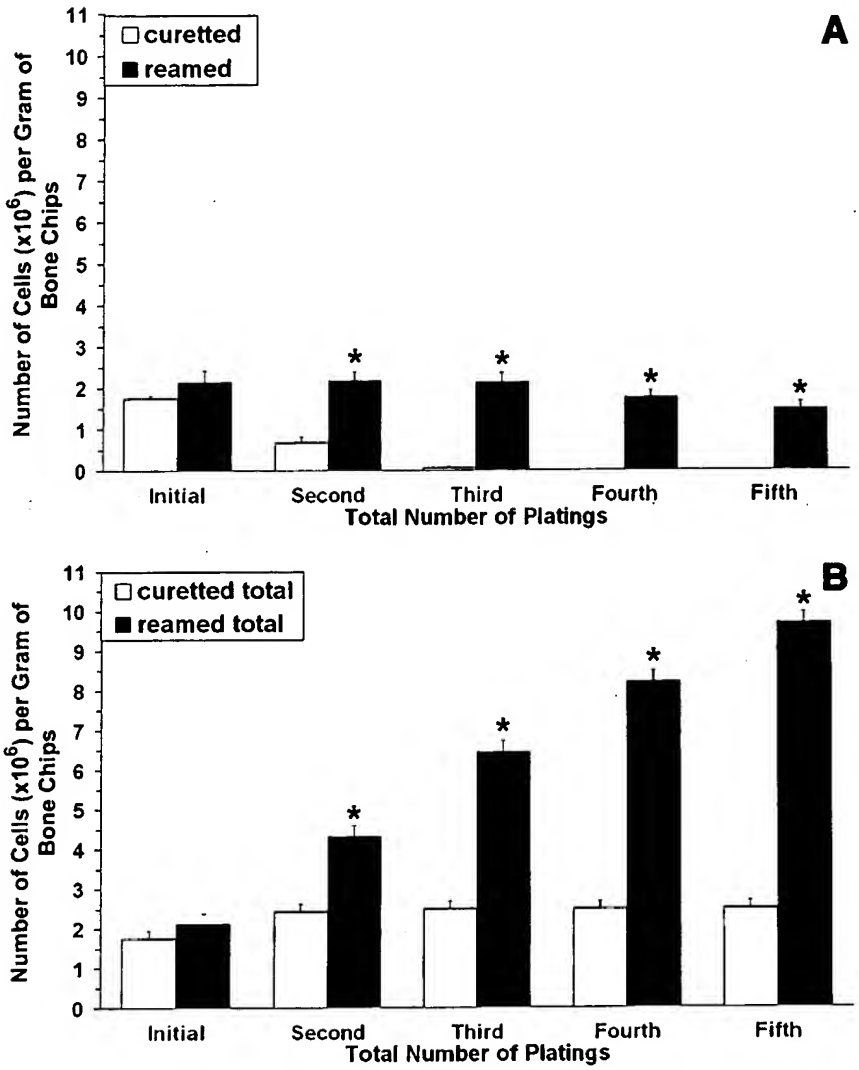


Fig. 2. Comparison of cell yields per gram of bone chips isolated with the curetting and reaming techniques and subjected to a total of five consecutive platings. Number of cells isolated at each plating (**A**); total number of cells isolated after each plating (**B**). After 4 wk, initially reamed primary cultures generated a mean of 2.150×10^6 cells/g of bone chips as compared to 1.75×10^6 cells/g of curetted fragments. Upon subsequent replating, the curetted bone chips produced significantly smaller quantities of cells, with completely diminished production by the fourth plating. This was in contrast to cells from the reamed bone, which repeatedly attained near confluence, reaching a total of $9.68 \times 10^6 \pm 0.324 \times 10^6$ cells after five platings as compared to $2.93 \times 10^6 \pm 0.189 \times 10^6$ cells derived from curetted cultures (* $p \leq 0.05$).

genes were not expressed. In contrast, after cell culture in the presence of osteogenic supplements for 21 d, RNA expression of alkaline phosphatase, bone sialoprotein, collagen type I, and osteocalcin was readily detectable. Moreover, the levels of mRNA expression of these genes were similar in the two types of osteogenic cultures.

3.1.4. Alkaline Phosphatase Histochemistry

Alkaline phosphate staining (**Fig. 4**) of the osteogenic cell cultures isolated through the two procedures used in the reverse transcription (RT)–PCR analysis showed enzyme activity levels that were in agreement with the mRNA data. Cell cultures treated with osteogenic supplements for 10 d

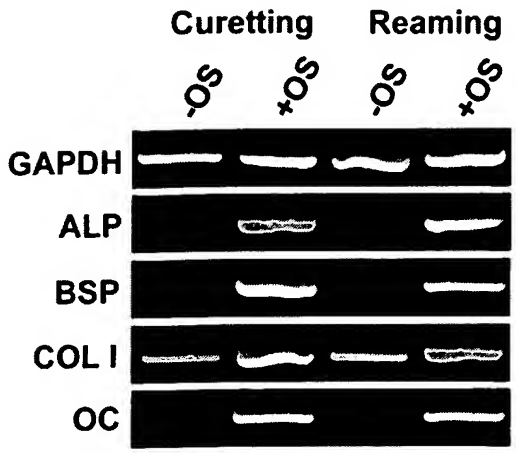


Fig. 3. Osteogenic nature of trabecular bone-derived cells. The mRNA was obtained from untreated cells (+OS) as well as from cells treated with osteogenic supplements (+OS) for 21 d, to compare osteogenic gene expression with the two isolation procedures. Cells from the two isolating procedures responded similarly to the supplements by transcriptionally upregulating genes associated with osteoblast differentiation.

showed a marked increase in the level of alkaline phosphatase staining as compared with the corresponding untreated controls. However, minimal differences were evident between cells isolated by the two procedures.

3.2. Viability and Proliferation of Cells Derived from Replated Chips

Cells obtained from different platings of reamed trabecular bone fragments were compared on the basis of their morphology and proliferation at 8 h and d 10 and 20 (**Fig. 5**). At 8 h after plating, cultures were examined for the presence of residual attached cells after thorough pre-treatment with collagenase in the case of the initial plating, or after extensive washings in the case of replated chips. No attached cells were present in any of the three cultures (**Fig. 5A–C**). Microscopic observation of initial and replated bone chip-derived cell cultures at d 10 (**Fig. 5D–F**), revealed small, fibroblastic, semiflattened cells with very similar patterns of migration and growth. This similarity was still evident on d 20 (**Fig. 5G–I**), with cells attaining near confluence in all three cultures. Results for the cell proliferation MTS assays (**Fig. 6**) showed similar A_{490} values for all three arthroplasty specimens through 24 d of culture; these results supported the microscopic analysis, indicating that cell viability and proliferation were not compromised in cells obtained from the multiple replating of bone chips.

3.3. Multilineage Differentiation and Gene Expression

Trabecular bone fragments isolated through the reaming method were cultured until cells migrating from the chips achieved 70–80% confluence. After removal of the cells with trypsin, the bone fragments were put back into culture until new migrating cells attained confluence. This system was maintained for a total of five consecutive cultures. Confluent cells obtained from the fifth plating of the chips were either left undifferentiated or cultured in media permissive for differentiation along mesenchymal lineages, namely osteogenesis, adipogenesis, and chondrogenesis (**Fig. 7**). Analysis of undifferentiated cells by RT-PCR showed constitutive expression of the collagen type I gene, but no expression of the other bone-, adipose-, or cartilage-specific genes. Cells treated with osteogenic supplements expressed the bone-specific genes *ALP*, *BSP*, and *OC*, and upregulated expression of the collagen type I gene. These osteogenically induced cells also expressed aggrecan in small amounts. Monolayer cultures maintained in supplemented medium conducive for adipogenesis expressed LPL and PPARγ2, as well as low levels of alkaline phosphatase and collagen type I. Additionally, cells cultured as high-density pellets with TGF-β1 supplementation for 21 d expressed the cartilage-specific collagen types II and IX genes, along with high levels of aggrecan and Sox 9. Also expressed were low levels of alkaline phosphatase and collagen type I transcripts.

4. Discussion

In this study we have introduced a simple, reproducible method for obtaining significant numbers of mesenchymal progenitor cells from human trabecular bone. Cells isolated through this proce-

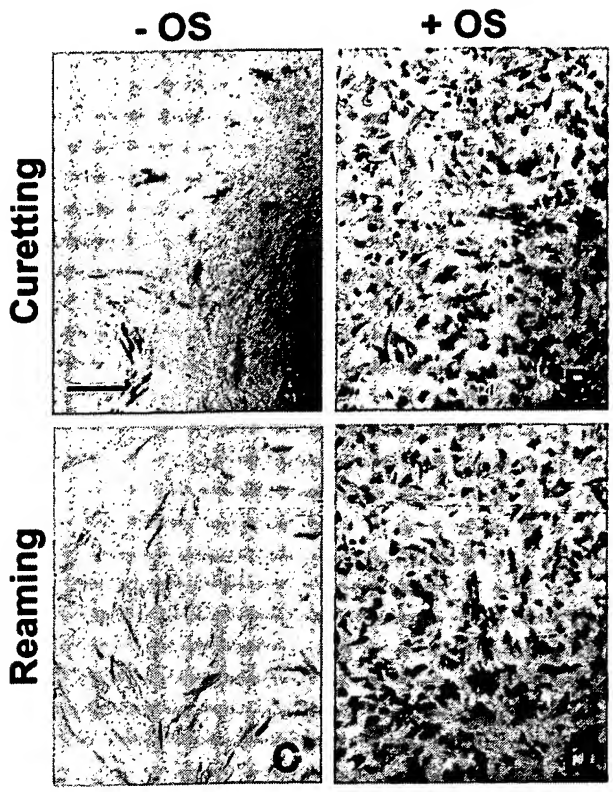
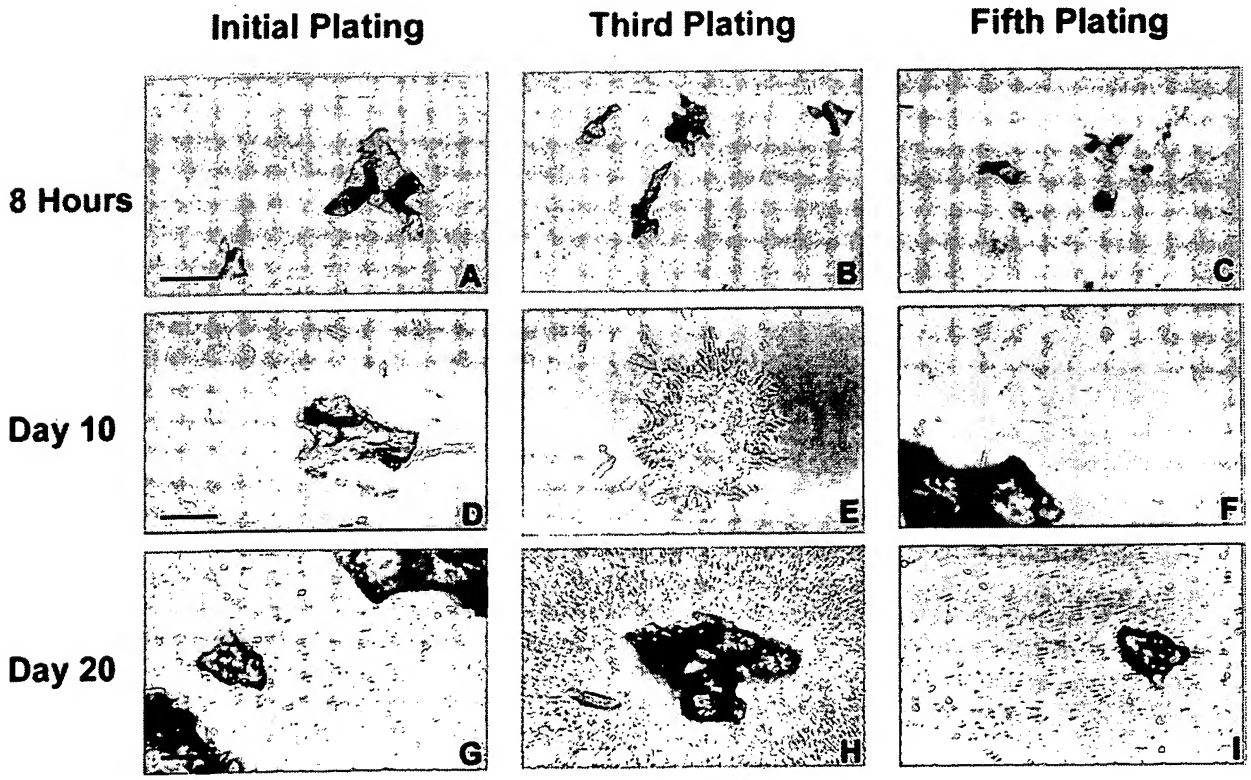


Fig. 4. *(left)* Alkaline phosphatase staining of cells from reamed (**A**,**B**) and curetted (**C**,**D**) bone explants. Cells were cultured with (+OS) or without (-OS) osteogenic supplements for 10 d. A similar, significant increase in alkaline phosphatase activity was seen in cells isolated with the two types of procedure upon osteogenic supplementation. Bar = 0.2 mm.

Fig. 5. *(below)* Isolation of mesenchymal progenitor cells by repeated plating of the same reamed trabecular bone fragments. Cells were examined at 8 h (**A–C**), and on d 10 (**D–F**) and d 20 (**G–I**) after plating of bone chips, and showed no evidence of contaminating cells. Cellular outgrowth and proliferation remained consistently similar through the initial (**A**,**D**,**G**), third (**B**,**E**,**H**), and fifth subsequent platings (**C**,**F**,**I**) with no apparent differences in cell morphology. **A–C**: bar = 0.4 mm. **D–I**: bar = 0.1 mm.



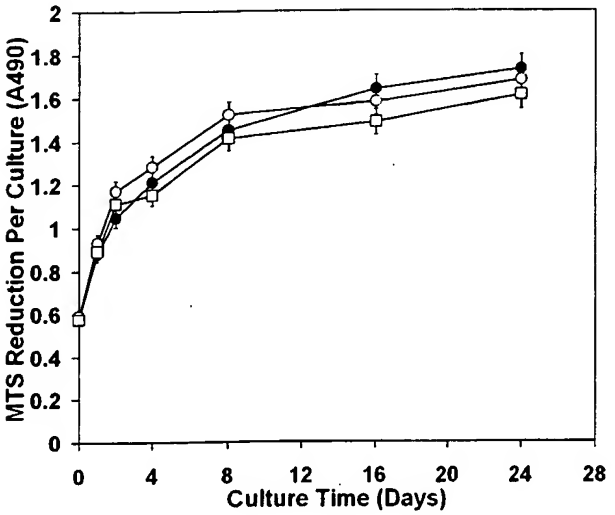


Fig. 6. Viability and proliferation of cells obtained from the initial (●), third (○), and fifth (□) explant plating of reamed trabecular bone fragments. Cultures were analyzed with the MTS assay, and comparisons were made with cultures established from the same patient, without significant differences being detected among the sequential cell isolates. In addition, the pattern of cell viability and proliferation was similar among cells isolated from the three arthroplasty specimens (data not shown).

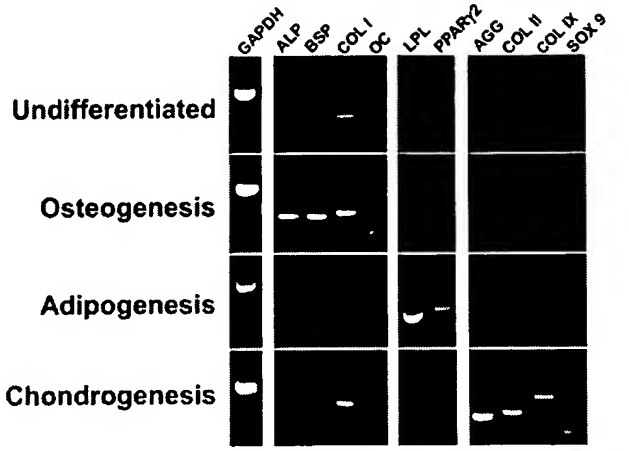


Fig. 7. RT-PCR analysis of the multilineage differentiation potential of trabecular bone-derived cells isolated from bone fragments through the reaming procedure and derived from the fifth explant replating. Expression of genes specific for osteogenesis, adipogenesis, and chondrogenesis (see **Table 1**) was analyzed with RT-PCR. Cells were left undifferentiated or were treated with supplements permissive for the indicated differentiation pathways as described under **Subheading 2.4.** Adipogenic cultures were analyzed after 2 wk of culture, osteogenic cultures on d 21, and chondrogenic pellet cultures on d 21. Results showed that the trabecular bone-derived cells responded to the differentiation conditions and expressed genes characteristic of the respective differentiation pathways.

dure, when provided with the appropriate cues, are capable of differentiating along osteogenic, adipogenic, and chondrogenic lineages. The differential expression of alkaline phosphatase activities in multiple subpopulations of primary osteoblasts suggests that these cells represent a heterogeneous population in different stages of differentiation *(22,23)*.

Although several procedures have been reported for isolating cells derived from adult human trabecular bone, the origins of the cell populations obtained remain to be identified. Most frequently, explant methods *(24–26)* and direct collagenase release methods *(27,28)* are used to yield osteoblast-rich populations; however, contamination by foreign marrow cells, especially fibroblasts, often predominates in the resulting cultures *(8)*. Collagenase pretreatment, followed by extensive washing, results in trabecular bone fragments completely devoid of surface-adherent cells, and with all soft-tissue elements removed *(8)*. When cultured, cells grow out from within these "bare bone" explants, leading to a homogeneous culture of cells with minimal cross-contamination. Although they remain to be completely characterized, these emerging cells are generally thought to display the characteristics of osteoblasts *(29)*. It has been postulated that these cells are actually osteocytes that have once again become mitotic, but proof of this is currently unavailable *(29)*. Another suggestion is that these cells are actually vasculature-associated cells such as pericytes, which have previously been reported to have osteogenic and limited chondrogenic potentials *(30–34)*. It has also been proposed that these trabecular bone-derived cells are bone-marrow-derived mesenchymal stem cells that have either lodged themselves within the bone fragments or have somehow escaped the collagenase digestion process. The procedure described

here most likely disqualifies the latter theory, since the small, thin bone fragments obtained in the procedure are extensively agitated and disturbed by the reamer. This, followed by extensive washing, a 3–4 h collagenase pretreatment, and further washing makes it highly unlikely for any contaminating cell type to be left behind. Nonetheless, the cellular origin of these multipotential cells definitely warrants further study.

In our study, trabecular bone-derived cells isolated with this new procedure were compared in terms of their morphology, growth properties, and differentiation potential with cells obtained through an established isolation method to ensure certain similarities (**Figs. 1–4**). Microscopic analysis of the continuous stream of cells migrating from the bone explants revealed uniformly long, fibroblast-like cells, which became proliferative and established individual colonies. However, the rate at which this occurred differed, according to the culture method used. Cells emerged, migrated, and proliferated considerably more rapidly from the reamed bone fragments than from the curetted fragments. These differences can be attributed to several factors. First, the time required for the processing of reamed trabecular fragments is less than half that required with the curetting method, perhaps allowing more cells to remain viable. Second, the reamed chips are smaller, thinner, and have a larger surface area, which means that cells have a shorter distance over which to migrate from the chips, eventually leading to more colony-forming units and, subsequently, to a larger number of established colonies. Moreover, when provided with osteogenic supplements, cells isolated and cultured according to the described procedure formed a matrix consisting predominantly of collagen type I, which mineralized and produced all of the major noncollagenous bone proteins in accordance with the osteoblastic phenotype *(35)*. The significant increase in the levels of alkaline phosphate mRNA and protein production during the second week of differentiation provided evidence that the cells were shifting to a differential state (data not shown) *(36,37)*. Furthermore, the increased expression of bone sialoprotein, a late marker of bone differentiation in vivo, and of osteocalcin, an osteoblast-specific protein, confirmed the osteoblastic phenotype *(38)*.

Interestingly, these reamed trabecular bone fragments retained the ability to maintain cellular outgrowth through many subcultures. Through five consecutive cultures of these bone fragments, active cellular outgrowth and proliferation was observed in each culture at approximately 1–2 wk after plating the chips. Upon culture of these isolated cells for 4 wk, the rate of cell growth began to decrease as intercellular contact increased and confluence was reached. No apparent differences in cell morphology were observed through extensive subculturing, with cells maintaining their elongated, fibroblastic appearance, although the cells never attained the typical octagonal morphology characteristic of the mature osteoblast *(39)*. Overall, these reamed trabecular bone fragments were capable of producing, after five successive platings, more than three times the quantity of cells derived with the traditional method of culture, thereby clearly supporting our claim that this method, which requires minimum culture expansion, can be used to generate a significant number of mesenchymal progenitor cells.

Analysis of lineage-specific gene expression by RT-PCR for the osteogenic, adipogenic, and chondrogenic differentiation of trabecular bone-derived cells from replated bone chips showed no differences in band intensity of the PCR products when subcultures were compared (data not shown). Moreover, cells from the fourth subculture retained their ability to differentiate along these mesenchymal lineages, with no significant variation in the levels of differentiation-specific gene expression. It is noteworthy that we used differentiation conditions that were previously established for bone marrow-derived mesenchymal stem cells to promote differentiation of the trabecular bone-derived cells in our study; in this mannner, these cells are comparable to the marrow stroma-derived cells *(18)*. Considering the wide array of reported sources for mesenchymal progenitor cells *(40–43)*, selection of an easily obtainable and accessible tissue source, capable of generating a clinically significant quantity of cells, becomes imperative. Currently, bone mar-

row aspirate is considered to be the most readily available source of mesenchymal progenitors. Muschler et al. *(44,45)* have established that the volume of bone-marrow aspirate taken from any site must not exceed 2 mL in order to maximize the concentration of mesenchymal progenitor cells obtained for subsequent expansion through tissue culture. It is thus instructive to compare the yield of mesenchymal stem cells from an equivalent volume of trabecular bone processed as described here with that from the direct plating of bone marrow-derived mesenchymal stem cells, a method that has been shown to allow a greater yield of cells than density-gradient fractionation *(16)*. Accordingly, a nearly equivalent cell yield is seen upon 4 wk of primary in vitro culture. This is assuming a bone mineral density of 1.0 g/cm^3 at the iliac crest, but depends greatly upon the site of cancellous bone extraction *(46–49)*. Moreover, in view of the potential yield of cells obtained by the successive replating of bone chips, trabecular bone processed according to the procedure described should be considered an alternative and desirable source of cells with multiple mesenchymal differentiation potential.

In conclusion, we have introduced a simple and reliable procedure for isolating large numbers of primary, mesenchymal progenitor cells. Cell populations isolated and cultured in the manner described here yield a clinically significant number of cells, and should be applicable to the engineering of mesodermally derived tissues for eventual therapeutic application.

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Original Article

Characterization of Multipotential Mesenchymal Progenitor Cells Derived from Human Trabecular Bone

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ABSTRACT

The in vitro culture of human trabecular bone-derived cells has served as a useful system for the investigation of the biology of osteoblasts. The recent discovery in our laboratory of the multilineage mesenchymal differentiation potential of cells derived from collagenase-treated human trabecular bone fragments has prompted further interest in view of the potential application of mesenchymal progenitor cells (MPCs) in the repair and regeneration of tissue damaged by disease or trauma. Similar to human MPCs derived from bone marrow, a clearer understanding of the variability associated with obtaining these bone-derived cells is required in order to optimize the design and execution of applicable studies. In this study, we have identified the presence of a CD73$^+$, STRO-1$^+$, CD105$^+$, CD34$^-$, CD45$^-$, CD144$^-$ cell population resident within collagenase-treated, culture-processed bone fragments, which upon migration established a homogeneous population of MPCs. Additionally, we have introduced a system of culturing these MPCs that best supports and maintains their optimal differentiation potential during long-term culture expansion. When cultured as described, the trabecular bone-derived cells display stem cell-like capabilities, characterized by a stable undifferentiated phenotype as well as the ability to proliferate extensively while retaining the potential to differentiate along the osteoblastic, adipocytic, and chondrocytic lineages, even when maintained in long-term in vitro culture. *Stem Cells 2003;21:681-693*

INTRODUCTION

The in vitro culture of human trabecular bone-derived cells has served as a useful system for the investigation of the biology of osteoblasts and has also provided insights into the interactions between bone and biomaterial for orthopedic and dental implants, their response to different growth factors and hormones, cell-matrix interactions, and osteoblast differentiation and maturation [1-9]. Our recent discovery of the multilineage mesenchymal differentiation potential of primary cell cultures derived from collagenase-treated human

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trabecular bone fragments [10] has prompted further interest in these cells in view of the potential application of mesenchymal progenitor cells (MPCs) in the repair and regeneration of tissue damaged by disease or trauma [11, 12]. Moreover, the identification and maintenance of MPCs in the undifferentiated phenotype depend on efficient methods of isolation as well as optimal conditions for subsequent culture in vitro, such as the tissue culture substrate, supplementation with proliferative and differentiation factors, and specific culture media. As such, establishing an optimal, standardized cell culture system is of critical importance, given the inherent complications associated with comparing studies that use a variety of different methodologies to obtain results. We have previously reported a simple, high-yield procedure that allows the isolation and production of a significant number of these MPCs from human trabecular bone, with improved yields over MPCs aspirated from bone marrow [13]. However, the origin, identification, characterization, and behavior of these trabecular bone-derived cells during primary and extensive subcultivation have yet to be provided.

As is the case for bone marrow-derived human MPCs, an understanding of the variability associated with the isolation of these cells is crucial for optimizing the experimental design of studies that utilize them. For example, techniques have been established to standardize the quantity of bone marrow aspirate of MPCs, the site and methodology of aspiration itself, the yield of progenitor cells identified by cell surface immunophenotyping, and their in vitro self-renewing capacity and multilineage differentiation potential following extensive subcultivation [14-17]. These known properties have permitted the utilization of MPCs in cell-matrix composites for various connective tissue engineering applications involving bone, adipose, cartilage, tendon, and muscle [12].

With this in mind, we have sought to characterize the native and culture-expanded trabecular bone-derived cell population and describe and optimize the proliferative and differentiative capacity of these cells during long-term in vitro culture. Specifically, we have compared the performance of two basal media, Dulbecco's modified Eagle's medium (DMEM), widely used for the culture of various cell types including bone marrow-derived MPCs [14, 18, 19], and DMEM with Kaighn's modification of Ham's F12 (F12K) supplemented with ascorbic acid and L-glutamine, which has been routinely used in studies involving cells derived from trabecular bone [9, 20, 21].

Materials and Methods

Isolation of Human Trabecular Bone-Derived Cells

Otherwise normal trabecular bone was obtained from the femoral heads of five patients (ages 51-78 years) undergoing total hip arthroplasty as a result of primary osteoarthritis, and processed using a rapid, high-yield protocol recently established in our laboratory [13] and approved by the Institutional Review Board of Thomas Jefferson University. Culture-processed trabecular bone fragments were subsequently plated in either A) DMEM (high-glucose and L-glutamine; Mediatech, Inc.; Herndon, VA; http://gomediatech.com) supplemented with 10% fetal bovine serum (FBS, Premium Select, Atlanta Biologicals; Atlanta, GA; http://www.atlantabio.com) from selected lots [22] and 50 μg/ml penicillin-streptomycin, or B) calcium-free DMEM-F12K (Specialty Media; Phillipsburg, NJ; http://www.cmt-inc.net) supplemented with 10% FBS from the same selected lots, 50 μg/ml ascorbate, 2 mM L-glutamine, and 50 μg/ml penicillin-streptomycin. Medium was changed every 3-4 days. Subconfluent monolayers of cells were removed with 0.25% trypsin containing 1 mM EDTA (GIBCO/BRL, Life Technologies; Grand Island, NY; http://www.invitrogen.com) and utilized for study or serially passaged at a ratio of 1:3. Primary cells that were initially cultured in calcium-free DMEM-F12K were switched to medium with normal amounts of calcium upon subculture.

Immunofluorescence Analysis of Human Trabecular Bone Chips and Cell Monolayers

Collagenase-treated human trabecular bone chips were harvested following 0, 7, and 14 days of culture; decalcified for 14 days using 0.38 M EDTA; fixed overnight at 4°C in 4% phosphate-buffered paraformaldehyde (FD Neuro Technologies, Inc.; Baltimore, MD; http://www.fdneurotech.com); embedded in paraffin; and sectioned at a thickness of 8 μm. STRO-1 was detected using a mouse IgM primary antibody (Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA; *Dr. B. Torok-Storb*). CD105 was detected using a mouse IgG_1 primary antibody (BD Transduction Laboratories; San Diego, CA; http://www.bdbiosciences.com); CD73 was detected using an R-phycoerythrin-conjugated mouse primary antibody (PharMingen; San Diego, CA; http://www.bdbiosciences.com/pharmingen). CD45 was detected using a PerCP-Cy5.5-conjugated mouse IgG_1 primary antibody (BD Biosciences; San Jose, CA; http://www.bdbiosciences.com). CD34 was detected using a monoclonal mouse IgG_1 primary antibody and CD144 (VE-cadherin) was detected using a polyclonal rabbit primary antibody (Zymed Laboratories, Inc.; San Francisco, CA; http://www.zymed.com). The sections were incubated in the following secondary antibodies: for STRO-1, fluorescein-conjugated goat anti-mouse IgM (Vector Laboratories, Inc.; Burlingame, CA; http://www.

vectorlabs.com); for CD105, tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG was used; for CD34, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse was used; for CD144, FITC-conjugated goat anti-rabbit was used. All sections were Hoechst Dye 33342 (Molecular Probes; Eugene, OR; http://www.probes.com) stained. Primary cultures of cell monolayers, fixed in 2% phosphate-buffered paraformaldehyde, were treated as described above.

Determination of Colony-Forming Unit-Fibroblast (CFU-F) Frequency

The initial cell population that exited the culture-processed trabecular bone fragments was trypsinized as described above and replated as single cells at a density of 100 cells per well of a 24-well plate in either DMEM or DMEM-F12K. The total number of cells that replicated to form colonies was subsequently determined following 7 days of culture ($n = 416$ and 400, respectively).

Differentiation of Cell Cultures along Mesenchymal Lineages

Osteogenic induction of confluent monolayer cultures was accomplished using DMEM supplemented with 10% FBS, 50 μg/ml ascorbate, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone for 21 days, with medium changes every 3-4 days [17]. Adipogenic differentiation was induced using DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μg/ml insulin for 14 days [17]. Control cultures were maintained without osteogenic or adipogenic supplements, respectively. Chondrogenesis of high-density pellet cultures (2×10^5 cells/pellet, $500 \times g$ for 5 minutes) was induced using serum-free DMEM supplemented with 50 μg/ml ascorbate, 0.1 μM dexamethasone, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate, and ITS-plus (Collaborative Biomedical Products; Cambridge, MA; http://www.bioscience.org/company/hbecton.htm) [18, 19, 23]. Recombinant human transforming growth factor-β1 (TGF-β1; R&D Systems; Minneapolis, MN; http://www.rndsystems.com) was also added to the chondrogenic induction medium at a final concentration of 10 ng/ml. Pellet cultures were maintained for 21 days with medium changes every 3-4 days. Control pellet cultures were maintained without the addition of TGF-β1.

Cell Proliferation Assay

Undifferentiated cells were added to a 96-well plate (1×10^3 cells/well) and assessed for cell viability and proliferation at days 1, 2, 4, 8, 16, and 24 using the CellTiter 96 AQueous One Solution MTS Cell Proliferation Assay (Promega; Madison, WI; http://www.promega.com) according to the manufacturer's protocol.

Population-Doubling Potential

For primary cultures, the initial number of cells that migrated from the bone chips and replicated to form colonies was counted, averaged from a total of five donors, and used to ascertain the number of population doublings based upon the total number of cells obtained at 80% confluency. For each subsequent passage, cells were seeded at a density of 10,000 cells/cm^2, and the population doublings were also calculated based upon the total number of cells obtained when 80% of the tissue culture polystyrene was covered with cells (approximately 4 weeks).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA was extracted using Trizol Reagent (GIBCO/BRL) according to the manufacturer's protocol. RNA samples were reverse transcribed using random hexamers and the SuperScript First Strand Synthesis System (GIBCO/BRL). PCR amplification of cDNA was carried out using AmpliTaq DNA Polymerase (Perkin Elmer; Norwalk, CT; http://www.perkinelmer.com) and the gene-specific primer sets listed in Table 1. These genes included the bone-specific genes, alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type Iα2 (Col Iα2), and osteocalcin (OC); the adipose-specific genes, lipoprotein lipase (LPL) and peroxisome proliferator-activator receptor-γ2 (PPARγ2); and the cartilage-specific genes, collagen types II (Col II), IX (Col IX), and XI (Col XI), aggrecan (AGN), and Sox 9. Thirty-two cycles were used for all genes and consisted of a 1-minute denaturation at 95°C, a 1-minute annealing at 57°C (Col II, Col IX, Col XI, AGN, and Sox 9) or 51°C (all remaining genes); a 1-minute polymerization at 72°C; and a final 10-minute extension at 72°C. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a control for RNA loading of samples. PCR products were analyzed electrophoretically using an ethidium bromide 2% MetaPhor agarose gel (BioWhittaker Molecular Applications; Rockland, ME; http://www.cambrex.com).

Alkaline Phosphatase Histochemistry

Osteogenic monolayer cultures were stained histochemically for alkaline phosphatase (Sigma Cat. No. 86-C; St. Louis, MO; http://www.sigmaaldrich.com) according to the manufacturer's protocol.

Oil Red O Histochemistry

Control and adipogenic monolayer cultures were fixed for 15 minutes using 2% phosphate-buffered paraformaldehyde (FD NeuroTechnologies), incubated in 60% isopropanol for 5 minutes, stained with Oil Red O for 5 minutes, rinsed with tap water, and counterstained with hematoxylin for 1 minute.

Table 1. RT-PCR primers for differentiation-specific gene expression analysis: sequence and expected product size

Gene	Primer sequences (5′-3′)		Position (bp)	Expected product size (bp)
Housekeeping gene				
GAPDH	Sense:	GGGCTGCTTTTAACTCTGGT	134-835	702
	Antisense:	GCAGGTTTTTCTAGACGG		
Bone-specific genes				
ALP	Sense:	TGGAGCTTCAGAAGCTCAACACCA	122-575	454
	Antisense:	ATCTCGTTGTCTGAGTACCAGTCC		
BSP	Sense:	AATGAAAACGAAGAAAGCGAAG	549-998	450
	Antisense:	ATCATAGCCATCGTAGCCTTGT		
Col Iα2	Sense:	GGACACAATGGATTGCAAGG	3,209-3,669	461
	Antisense:	TAACCACTGCTCCACTCTGG		
OC	Sense:	ATGAGAGCCCTCACACTCCTC	19-312	294
	Antisense:	GCCGTAGAAGCGCCGATAGGC		
Adipose-specific genes				
LPL	Sense:	GAGATTTCTCTGTATGGCACC	1,261-1,536	276
	Antisense:	CTGCAAATGAGACACTTTCTC		
PPARγ2	Sense:	GCTGTTATGGGTGAAACTCTG	153-503	351
	Antisense:	ATAAGGTGGAGATGCAGGCTC		
Cartilage-specific genes				
Col II	Sense:	CAGGTCAAGATGGTC	1,341-1,717	377
	Antisense:	TTCAGCACCTGTCTCACCA		
Col IX	Sense:	GAAAATGAAGACCTGCTGG	126-641	516
	Antisense:	GAAAAGGCTGCTGTTTGGAGAC		
Col XI	Sense:	GGAAAGGACGAAGTTGGTCTGC	90-679	590
	Antisense:	TTCTTCACGCTGATTGCTACCC		
Aggrecan	Sense:	TGAGGAGGGCTGGAACAAGTACC	6,591-6,910	350
	Antisense:	GGAGGTGGTAATTGCAGGGAACA		
Sox 9	Sense:	ATCTGAAGAAGGAGAGCGAG	548-811	264
	Antisense:	TCAGAAGTCTCCAGAGCTTG		

Alcian Blue Histochemistry

Cell pellet cultures, rinsed twice with phosphate-buffered saline (PBS), were fixed for 2 hours in 2% PBS-buffered paraformaldehyde, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, embedded in paraffin, and sectioned at an 8 μm thickness for histological staining with alcian blue (pH 1.0) [24].

Results

Immunofluorescence Analysis of Human Trabecular Bone Chips and Monolayer Cultures

Collagenase-treated, culture-processed trabecular bone fragments harbor cells distributed throughout the confines of the mineralized matrix tissue (Fig. 1). As a function of culture time, these cells, appearing mitotically active, migrated outward where they lined the periphery of the fragments (Fig. 1C, 1F, 1I, 1L, 1O, 1R). This migration time apparently coincided with the lag period of 1 week, as described below, required before cellular outgrowth from the explanted bone began. Additionally, all cells resident within the fragments detected by Hoechst stain also stained positively for CD73, STRO-1, and CD105, individually, but negatively for CD34, CD45, and CD144. Following approximately 3 weeks of culture, cells that had successfully migrated out from within the trabecular bone chips retained the CD73, STRO-1, and CD105 cell surface antigens, while remaining CD34, CD45, and CD144 negative. There were no cells in monolayer culture that did not stain positively for the CD73, STRO-1, or CD105 antigens or negatively for CD34, CD45, or CD144 (Fig. 2).

Cell Viability and Proliferation

Phase contrast microscopy of the trabecular bone fragments seeded in DMEM and DMEM-F12K culture media initially showed fragments devoid of soft tissue residue and contaminating cells (Fig. 3A, 3B). Upon culture, an initial lag

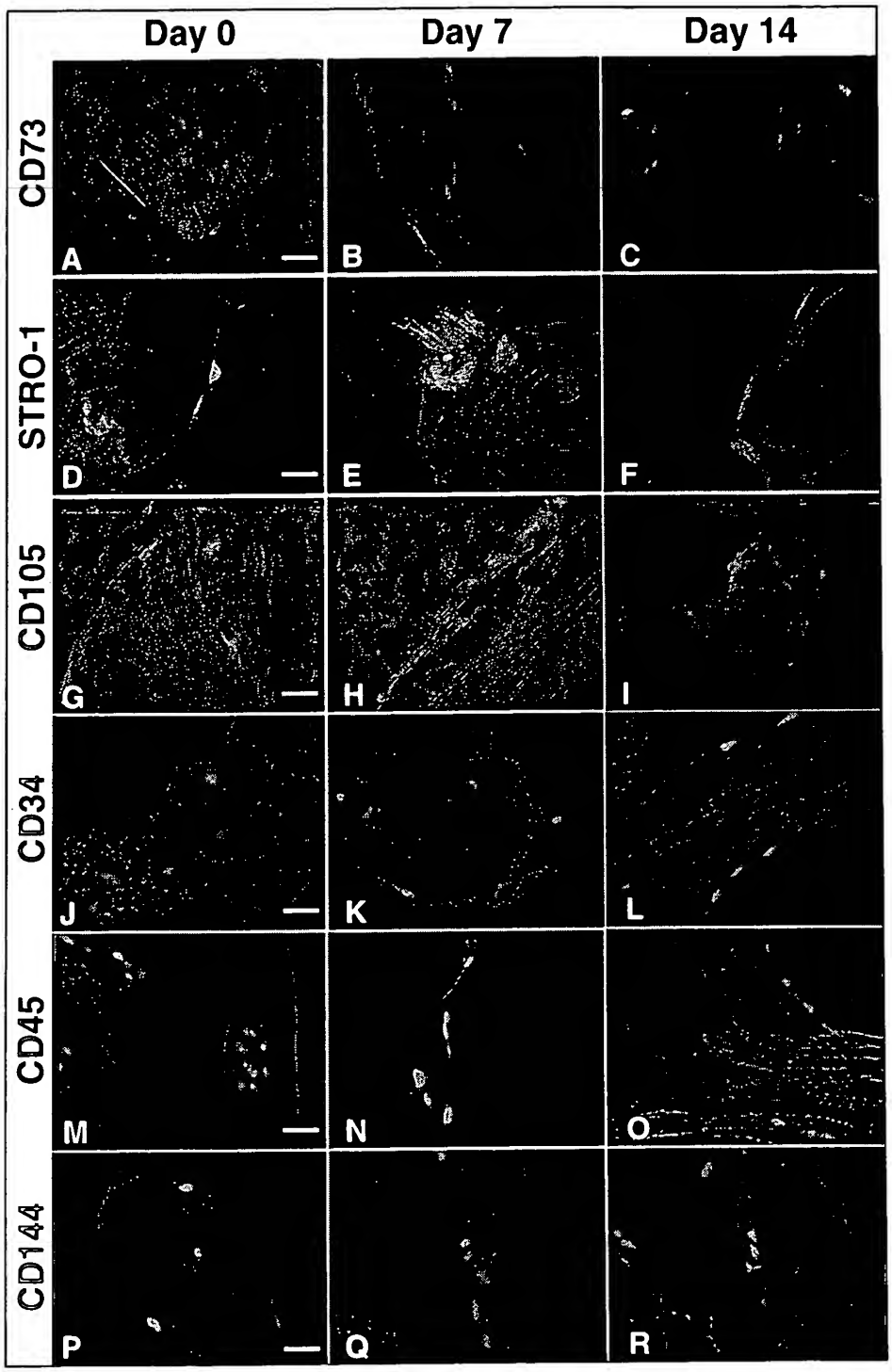

Figure 1. Collagenase-treated human trabecular bone chips harvested following 0, 7, and 14 days of culture and immunostained as described in Materials and Methods reveal cells that express the cell surface antigens CD73 (A-C, orange-red), STRO-1 (D-F, green), and CD105 (G-I, red), and fail to express CD34 (J-L), CD45 (M-O), and CD144 (P-R). Between days 7 and 14 of culture, most of the cells have migrated to the periphery of the bone fragments. Cell nuclei are counterstained with Hoechst Dye 33342. Bar = 20 μm.

phase of 4-6 days was seen in DMEM cultures, after which cells entered the log phase of growth and began to actively migrate from the bone chips and proliferate. This was in sharp contrast to the 10- to 12-day lag phase seen in DMEM-F12K cell cultures, followed by the slow, gradual cellular migration from the bone fragments (Fig. 3C, 3D). This difference was more apparent when cultures were observed microscopically on days 14 (Fig. 3E, 3F) and 21 (Fig. 3G, 3H), revealing the ability of DMEM cultures to attain a higher limiting cell density. Cellular morphology during the initial primary culture was similar in both populations, revealing homogeneous spindle-shaped cells.

The CFU-F assay was used to ascertain the colony-forming potential of trabecular bone-derived cells cultured in the two media, which were in the process of exiting the bone fragments. Following 3 days of culture in DMEM, approximately 73% of individual cells established small (4-8 cells) colonies, all of which proliferated and grew rapidly, forming large (100-200 cells) colonies by day 7. In contrast, only 48% of single isolated cells cultured in DMEM-F12K followed the same pattern of colony formation. Following increased culture time, a small percentage of the cells cultured in either medium that failed to yield colony formation during the first 7 days of culture began to proliferate and established latent colonies.

Cells serially passaged and grown in the two media followed patterns of growth similar to their respective primary cultures (Fig. 4). DMEM-F12K cell cultures experienced an initial lag phase of 1-3 days, followed by a log phase of 10-14 days, and a plateau phase of 4-6 days, after which mitotic division slowed dramatically (Fig. 4A-4C). In comparison, the growth curves of cells cultured in DMEM depict a minimal lag phase, followed by steeper log and

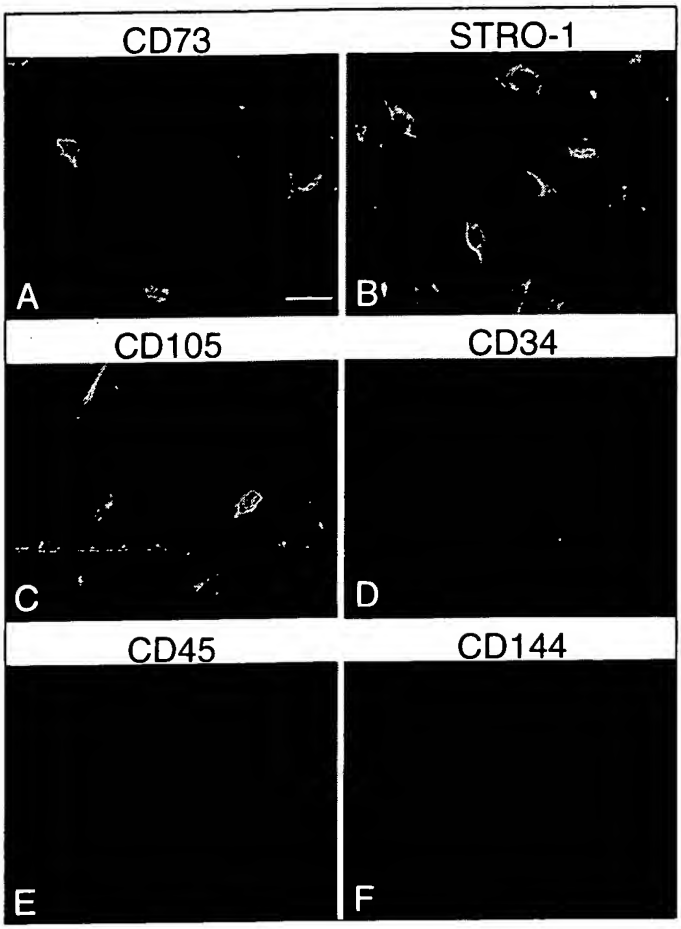

Figure 2. Cell monolayers derived from collagenase-treated trabecular bone chips uniformly express the CD73 (A, orange-red), STRO-1 (B, green), and CD105 (C, red) cell surface antigens. No cells expressing CD34 (D), CD45 (E), or CD144 (F) are present within the culture. Cell nuclei are counterstained with Hoechst Dye 33342. Bar = 20 μm.

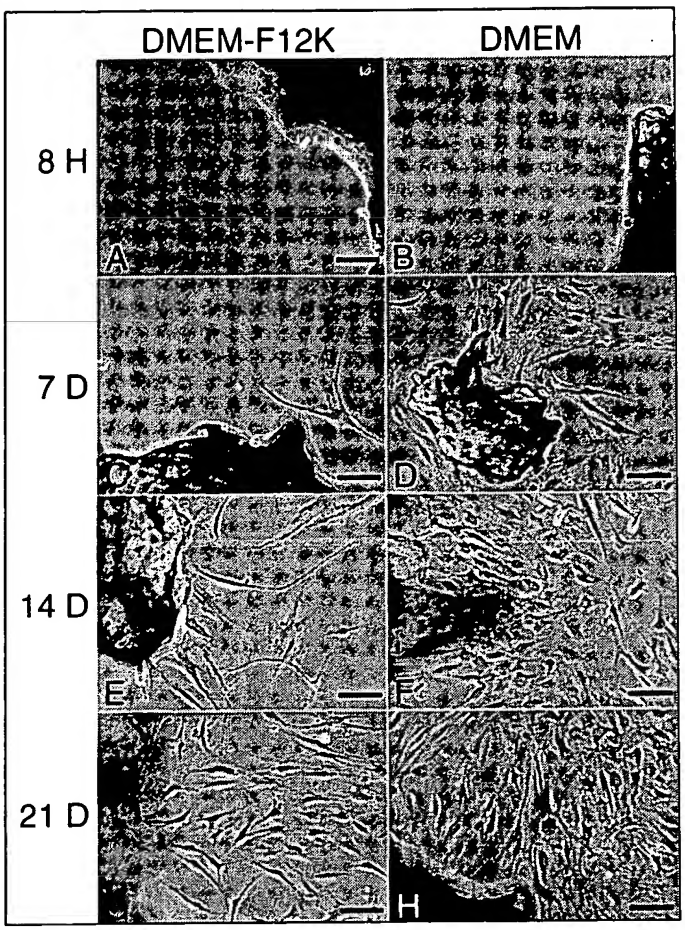

Figure 3. Morphology of reamed trabecular bone explants cultured in DMEM-F12K (A, C, E, G) and DMEM (B, D, F, H). Approximately 8 hours after plating, no attached cells can be seen contaminating the culture, and all bone fragments appear completely devoid of soft-tissue components (A, B). After 7 and 14 days, cells began to migrate and proliferate more rapidly from bone chips cultured in DMEM (C, D and E, F). Differences in the number of cells and colonies became more apparent following 21 days in culture (G, H). Bar = 25 μm.

plateau phases. As passage number increased, growth rates for DMEM-F12K cultures decreased, resulting in a smaller total number of cells, i.e., the log phase cell growth rate diminished more rapidly with passaging for the cells cultured in DMEM-F12K.

Moreover, cells in DMEM-F12K attained a broad, flattened morphological appearance, a cessation of mitotic activity, and an increased accumulation of cellular debris after an average of 6 passages, thereby signifying replicative senescence. On average, the cumulative population doublings for DMEM-F12K cultured cells were 21.9 ± 1.8, with 10.1 doublings taking place during the primary culture, accounting for 46.1% of the total, and two doublings occurring at each subsequent passage (Fig. 5). In comparison, the mean cumulative population doublings for cells cultured in DMEM was 43.6 ± 2.2 through an average of 13 passages. Analysis of primary DMEM cultures revealed an average of 12.4 population doublings, with 2.4 doublings per subsequent passage (Fig. 5); this difference can be partially attributed to the higher limiting density reached by the cell population cultured in DMEM.

Analysis of Osteogenic Differentiation

Confluent first and fifth passage cells maintained in monolayer and cultured in basal DMEM and DMEM-F12K medium served as controls for the analysis of osteogenic gene expression (Fig. 6A, 6C). While these control cultures expressed Col I mRNA at similar levels, none of the other osteogenesis-specific marker genes were expressed. On the other hand, upon culture in the presence of osteogenic supplements (OS) for 21 days, RNA expression of ALP, BSP, Col I, and OC was readily detectable in first and fifth passage cells. In the first passage, OS-treated DMEM cultures exhibited a significantly higher level of alkaline

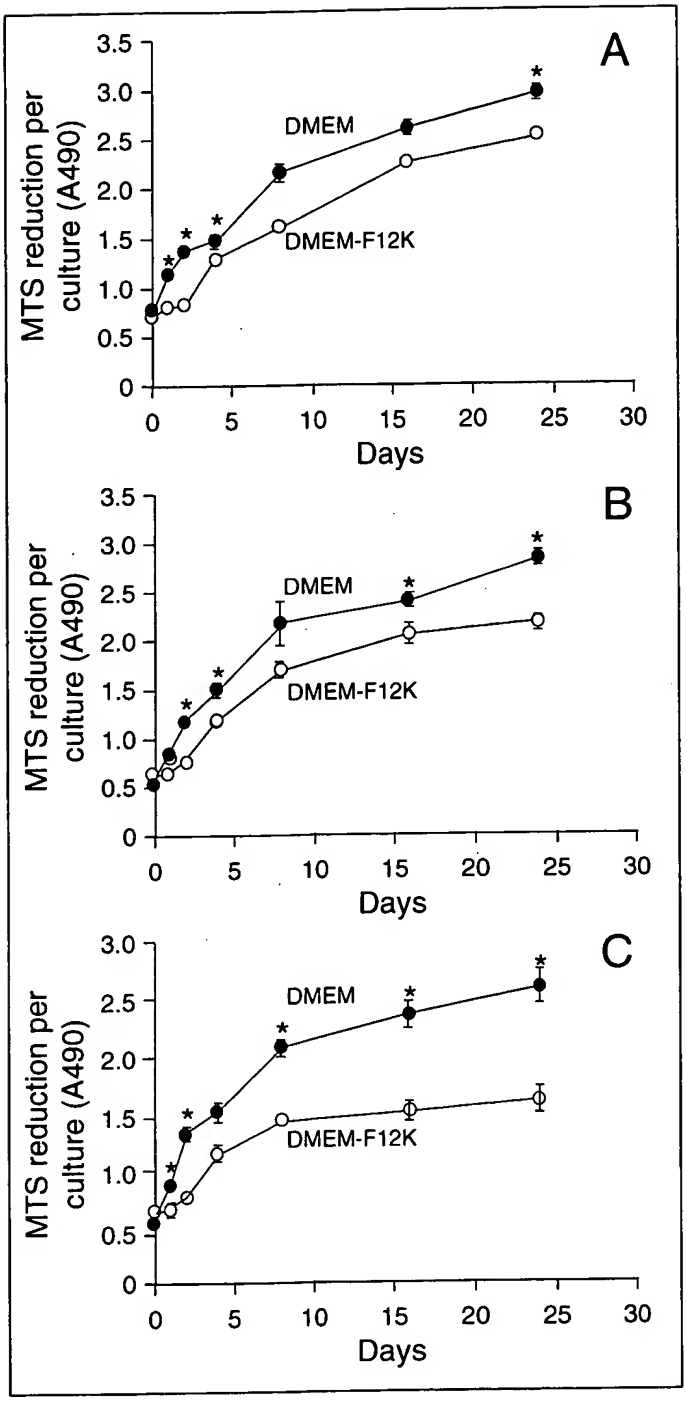

Figure 4. Viability and proliferation of cells grown in DMEM-F12K and DMEM, obtained from the first (A), third (B), and fifth (C) serial subculture. Cultures were established from the same patient and analyzed using the MTS assay (see Materials and Methods). Compared with DMEM cultures, DMEM-F12K cultures show a decrease in the slope of the log phase as a function of increasing passage. A similar pattern of cell viability and proliferation was seen among the three arthroplasty specimens (data not shown). The results represent the mean cell number (absorbance at 490 nm) ± standard deviation (n = 3). * p <0.05.

phosphatase gene expression as compared with similarly treated DMEM-F12K cultures, whereas the expression levels of BSP, Col I, and OC were equivalent (Fig. 6B). After serial subculture to passage five, significantly higher levels of ALP, BSP, Col I, and OC gene expression were seen in OS-treated DMEM cultures compared with similarly treated DMEM-F12K cultures (Fig. 6C, 6D). In all cases, the ALP enzyme activity levels were in direct agreement with the RT-PCR analysis of mRNA levels after 21 days of OS treatment (Fig. 7).

Analysis of Adipogenic Differentiation

Subconfluent monolayer cultures of first passage cells maintained in either DMEM or DMEM-F12K and cultured in media permissive for adipogenic differentiation for 14 days exhibited similar levels of LPL and PPARγ2 gene expression, whereas control cells treated without adipogenic supplements expressed significantly lower levels of LPL and failed to express PPARγ2 (Fig. 8A, 8B). Serial subculture appeared not to effect cells cultured in DMEM, as adipogenically induced cells of the fifth passage continued to express levels of LPL and PPARγ2 comparable to those of first passage cells (Fig. 8C, 8D). However, serial subculture appeared to significantly affect the adipogenic capacity of cells cultured in basal DMEM-F12K, as evidenced by fifth passage cells treated with adipogenic supplements, which expressed minimal amounts of LPL and failed to express PPARγ2, similar to the untreated controls. Oil Red O staining of similarly treated cultures was in accordance with adipogenic gene expression results (Fig. 9). As expected, control cultures maintained without adipogenic supplements did not stain positively for lipid droplets.

Analysis of Chondrogenic Differentiation

In the absence of TGF-β1, RT-PCR analysis showed constitutive expression of GAPDH in the first and fifth passage cells, but no cartilage-specific genes were expressed (Fig. 10). On the other hand, all cell pellets maintained in chondrogenic media and treated with TGF-β1 significantly upregulated the cartilage-specific genes, i.e., Col II, Col IX, Col XI, AGN, and Sox 9. These mRNA transcripts were readily detectable in both first and fifth passage cells treated with TGF-β1. First passage DMEM cultured cells maintained in high-density chondrogenic cultures with TGF-β1 exhibited a significantly higher level of Col II gene expression as compared with similarly treated DMEM-F12K cells, whereas expression of Col IX, Col XI, AGN, and Sox 9 was not significantly different between the two groups of cells (Fig. 10B). TGF-β1-treated cell pellets derived from fifth passage DMEM cultures expressed significantly higher levels of Col IX, AGN, and Sox 9, in addition to Col II as compared with similar passage DMEM-F12K medium cultures (Fig. 10C, 10D).

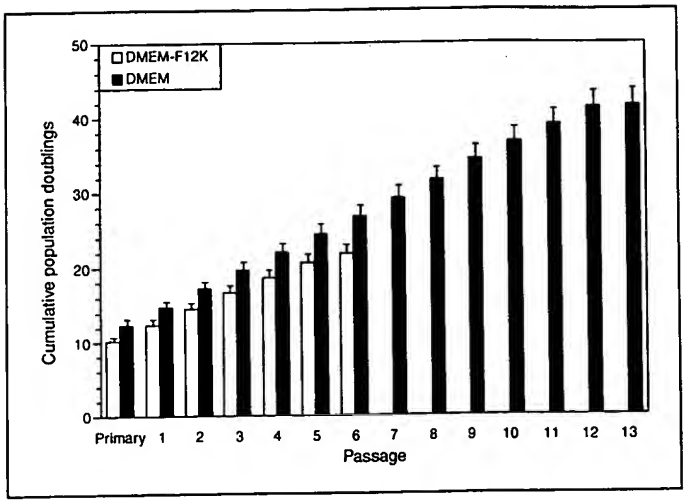

Figure 5. Population-doubling potential of trabecular bone-derived cells in two different culture media. The cumulative number of doublings was calculated from the initial establishment of colonies in primary culture through each subsequent passage until replicative senescence occurred. DMEM primary cultures (passage 0) underwent a total of 12.4 population doublings, as compared with 10.1 for DMEM-F12K cultures. Serially subcultured cells passaged at known densities underwent an average of 2.4 and 1.8 population doublings per passage in DMEM and DMEM-F12K cultures, respectively. Replicative senescence occurred after an average of five passages for DMEM-F12K cultures, while DMEM cultures continued to replicate until an average of 13 passages. The results represent the mean number of doublings ± SD of cells obtained from three donors.

As shown in Figure 11, 21-day TGF-β1-treated pellets derived from DMEM cultures of the first (D-F) and fifth (J-L) passage exhibited a dense, extensive, and organized sulfated proteoglycan-rich extracellular matrix surrounding chondrocyte-like cells, as compared with control pellets maintained without TGF-β1 (data not shown). The alcian blue staining intensity was higher than similarly treated pellets derived from DMEM-F12K cultured cells (A-C, G-I), which displayed a disorganized extracellular matrix devoid of the proteoglycan-rich characteristic of the in vitro chondrogenic phenotype. The pattern of the alcian blue histochemical staining was consistent with the mRNA gene expression of 21-day TGF-β1 pellet cultures.

Discussion

In this study, we have characterized in detail a population of cells resident within culture-processed human trabecular bone fragments that expressed STRO-1, CD73, and CD105, initially identified as antigens specific for nonhematopoietic bone marrow progenitor cells, and were negative for CD34, CD45, and CD144, common markers of hematopoietic and endothelial cells. The antibodies SH-2 and SH-3, and SH-4 [25], which recognize epitopes on CD105 [26] and CD73 [27], respectively, have also been shown to be nonreactive with the cell surfaces of osteoblasts and osteocytes, suggesting the presence of a population of multipotential cells distinct

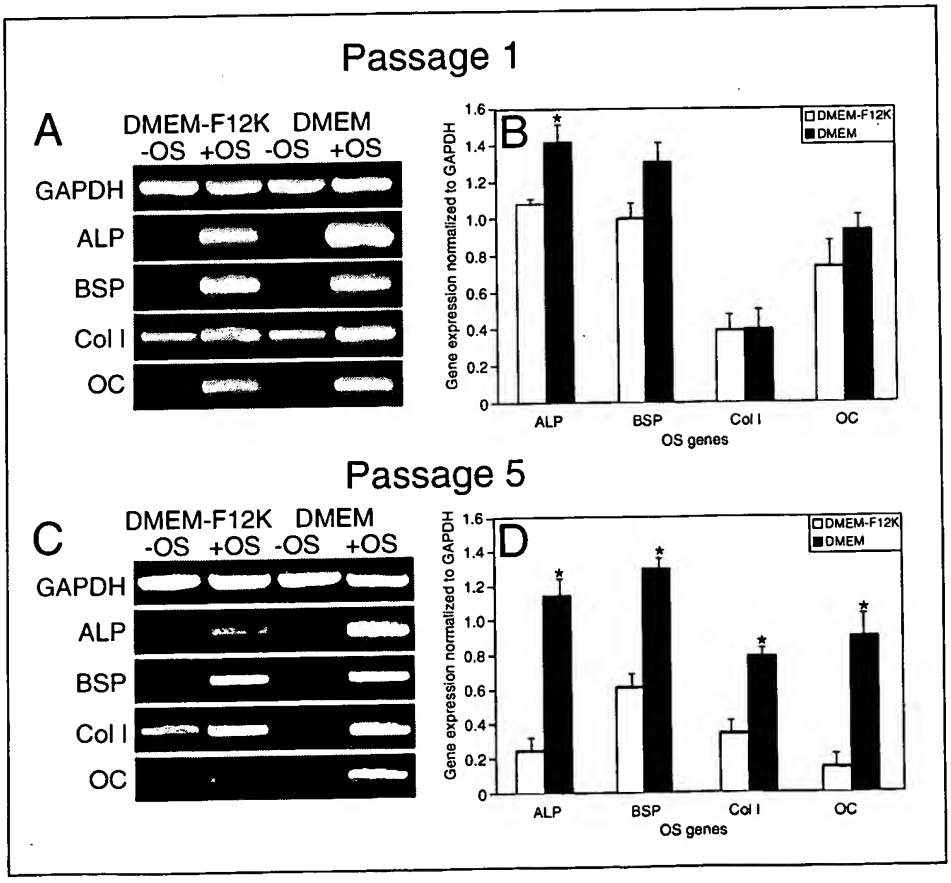

Figure 6. Osteogenic nature of serially passaged trabecular bone-derived cells cultured in DMEM and DMEM-F12K. The mRNA was obtained from untreated control cells (-OS) and from cells treated with osteogenic supplements (+OS) for 21 days, and analyzed by RT-PCR to compare osteogenic gene expression (ALP, BSP, Col I, OC). All RT-PCR products were fractionated by electrophoresis, and ethidium bromide intensities were quantified densitometrically. (A, B) Passage 1. (C, D) Passage 5. Although both cell cultures, DMEM and DMEM-F12K, responded osteogenically to the supplements during the first passage (A), the osteogenic capacity of DMEM-F12K cultures diminished significantly by passage 5. Moreover, DMEM cultures were more responsive to the OS supplements, indicated by the significant upregulation of ALP gene expression in the first passage (B), and of ALP, BSP, Col I, and OC in the fifth passage (D) upon OS treatment, compared with DMEM-F12K cultures.

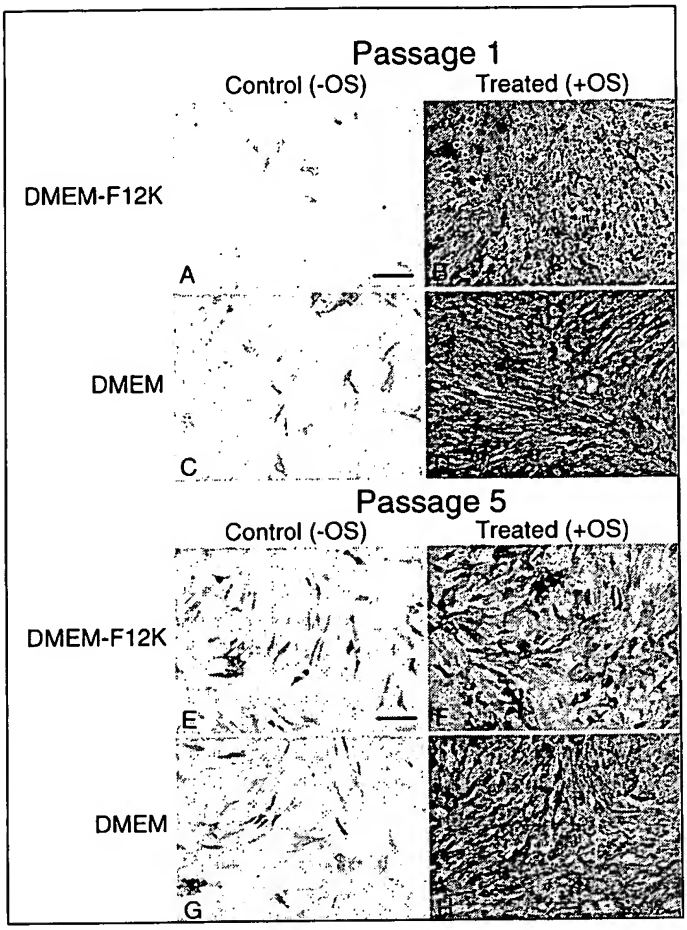

Figure 7. ALP activity of serially passaged cells grown in DMEM and DMEM-F12K, and then treated with (+OS) or without (–OS) osteogenic supplements for 10 days. (A-D) Passage 1; (E-H) Passage 5. Bar = 500 μm. DMEM cells (C, D and G, H) demonstrated a significant increase in ALP activity upon osteogenic supplementation for both Passage 1 and Passage 5 cultures, as did Passage 1 DMEM-F12K cultures (A, B). However, Passage 5 DMEM-F12K cultures (E, F) failed to respond to OS supplementation, as evidenced by unchanged staining intensity. Additionally, DMEM cultures displayed a comparatively higher level of ALP activity during each passage.

from other cell types normally associated with and present within trabecular bone. It is noteworthy that not all cells resident within the bone fragments display these cell surface epitopes, i.e., the multipotential cells are a subset of cells of the trabecular bone. Following migration from within the explants, these cells, which retain the same cell surface profile, proceeded to establish colonies. Analysis of the cell surface antigens of confluent monolayer cultures revealed a highly homogenous population of cells expressing CD73, STRO-1, and CD105, all previously shown to be associated with cells capable of differentiating along multiple mesenchymal lineages [26-29]. It is noteworthy that, in comparison, bone marrow-derived MPCs contain considerably fewer cells expressing these particular antigens than trabecular bone-derived cells, suggesting the presence of a significantly larger population of MPCs isolated from trabecular bone. Additionally, no cells expressing CD34, CD45, or CD144 were found within or associated with the trabecular bone fragments, indicating lack of any hematopoietic or endothelial cell contamination.

Analysis of cultures from all patients revealed that DMEM was significantly more effective in stimulating cellular outgrowth from the bone chips and promoting

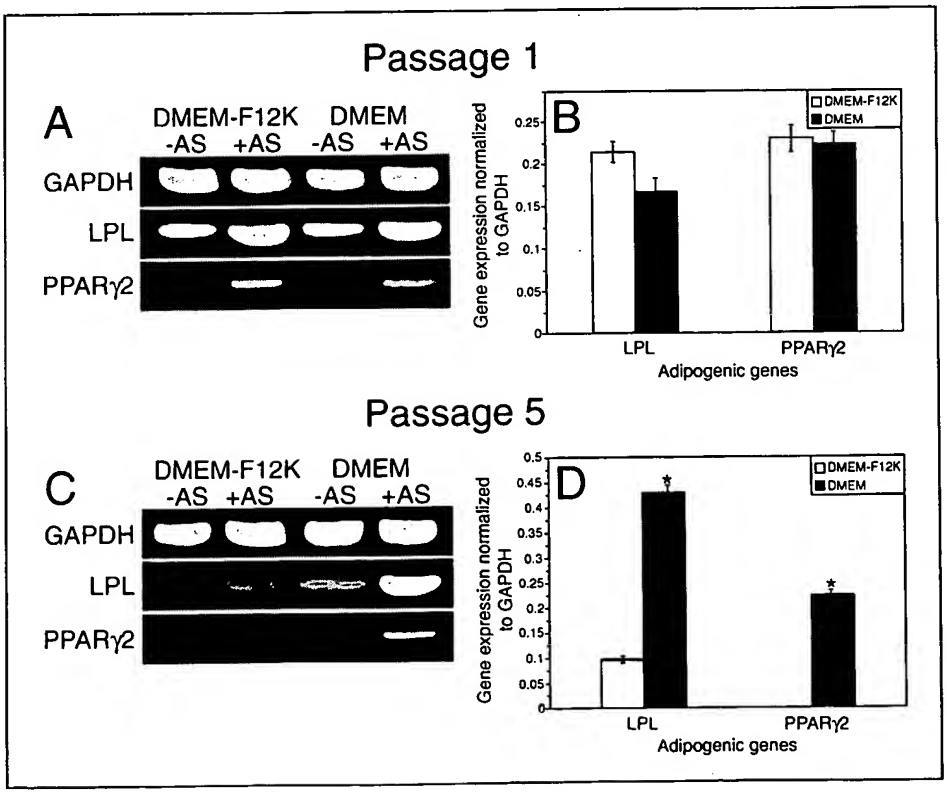

Figure 8. Adipogenic induction of serially passaged trabecular bone-derived cells cultured in DMEM and DMEM-F12K. The mRNA from untreated control cells (-AS), as well as cells treated with adipogenic supplements (+AS) for 14 days, were compared for LPL and PPARγ2 gene expression. First passage cells cultured in either basal medium were capable of adipose-specific gene expression (A, B); however, the adipogenic capacity of DMEM-F12K cultures rapidly diminished by passage 5 (C, D). In contrast, passage 5 DMEM cultures continued to respond to adipogenic supplements by upregulating LPL and PPARγ2 gene expression.

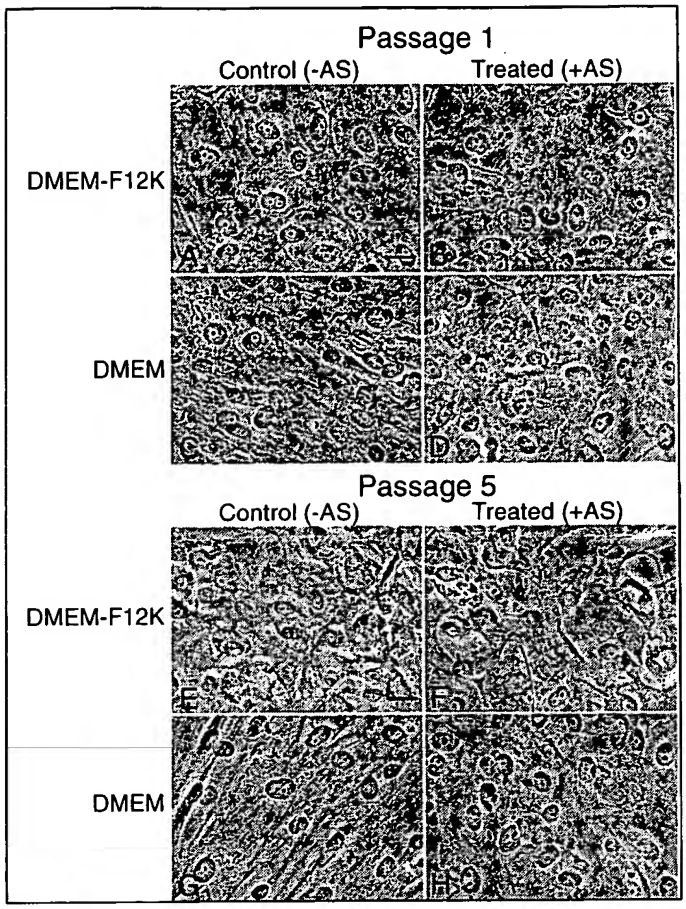

Figure 9. Oil Red O staining of serially passaged trabecular bone-derived MPCs cultured in DMEM or DMEM-F12K basal medium and treated with (+AS) or without (–AS) adipogenic supplements for 14 days. Similar to RT-PCR results, passage 1 cells cultured in either basal medium responded to AS, as evidenced by the accumulation of cytoplasmic lipid droplets (A-D). Passage 5 DMEM cultures responded similarly when induced (H). However, serially subcultured DMEM-F12K cultures could not be adipogenically induced beginning at passage 5, as evidenced by absence of stain (F). Bar = 20 μm.

the establishment of colonies as compared with DMEM-F12K cultures. However, the growth of individual colonies throughout primary culture in either medium appeared to occur at different rates. Some cells appeared to begin dividing immediately upon migration from the bone fragment, and others failed to yield colonies at all or did so only after several days in culture. Previous studies have shown that fibroblasts, which are capable of quickly adhering and establishing rapidly growing colonies, undergo an increased number of total population doublings as compared with cells that slowly divide and form smaller colonies [30]. Our data support previous research into the aging characteristics of bone marrow stromal cells, where the confirmed presence of two types of adherent cells in primary culture, a rapidly dividing spindle-shaped cell type and a much slower dividing broad and flattened cell type, was observed [14, 31]. A much larger proportion of the former cell type was present in DMEM primary cultures as compared with DMEM-F12K cultures. Moreover, the proportion of spindle-shaped cells to flattened cells began to decrease as a function of

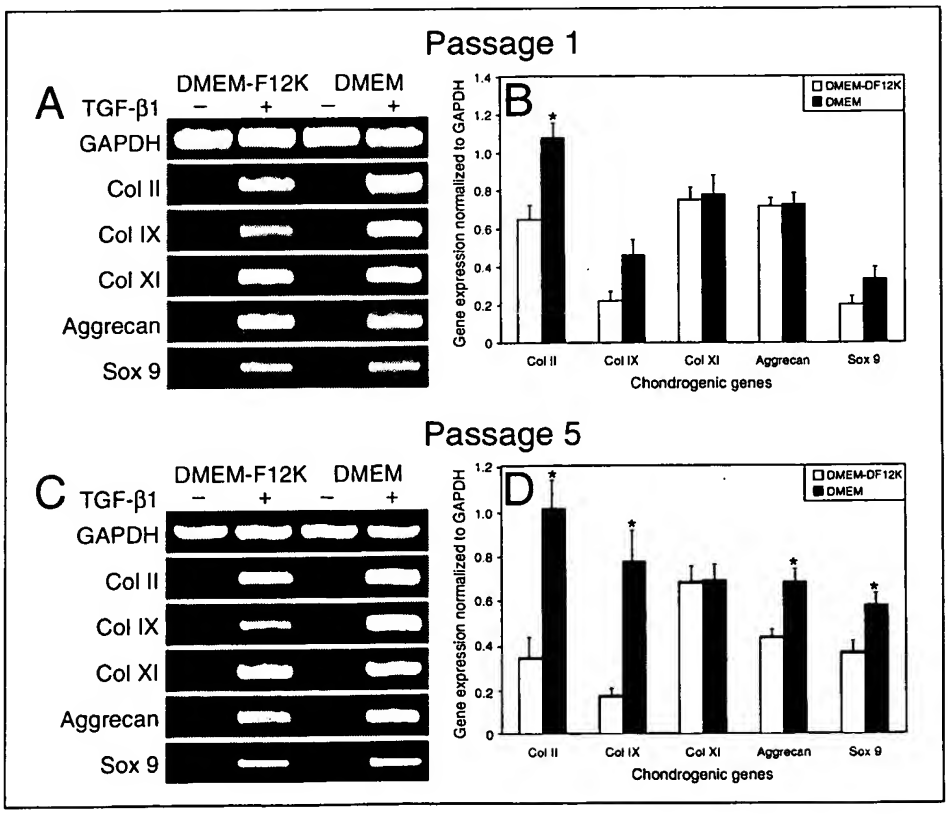

Figure 10. Chondrogenic differentiation of serially passaged cells grown in DMEM and DMEM-F12K (A, B) Passage 1; (C, D) Passage 5. Cells were initially cultured in either DMEM or DMEM-F12K medium, followed by chondrogenic induction. The mRNA was obtained from pellet cultures maintained with (+) or without (–) TGF-β1 for 21 days, and analyzed by RT-PCR to compare chondrogenic gene expression (Col II, IX, and XI, aggrecan, and Sox 9). RT-PCR products were fractionated by electrophoresis, and ethidium bromide intensities were quantified densitometrically. All treated cultures responded chondrogenically; however, the DMEM cultures were more responsive to chondrogenic induction as indicated by significant upregulation of Col II during the first passage (B), and Col II and XI, aggrecan, and Sox 9 during the fifth passage (D) compared with similarly treated DMEM-F12K cultures.

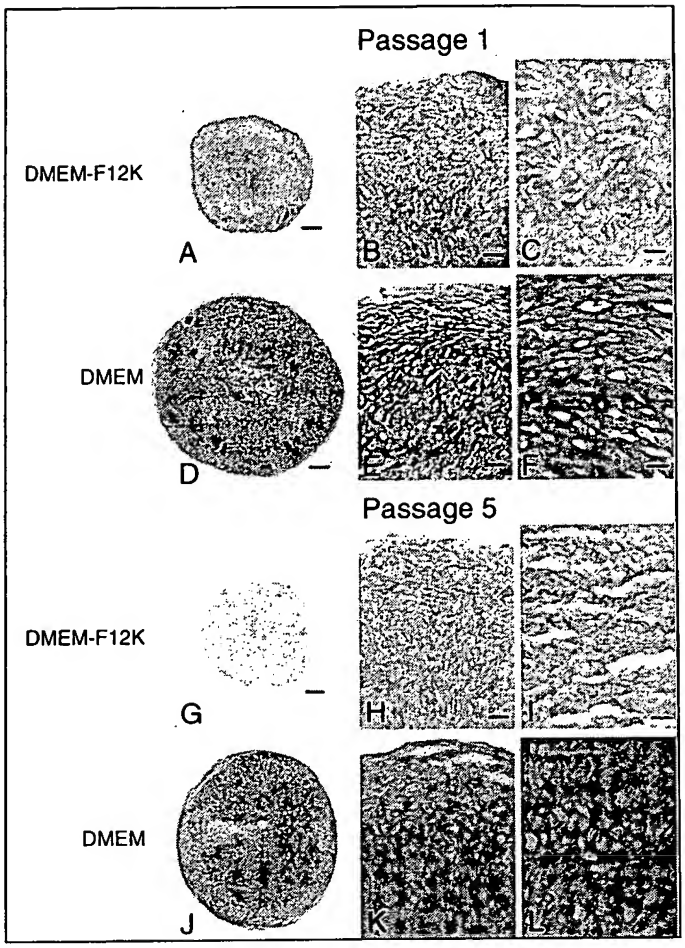

Figure 11. Alcian blue staining of serially passaged cells grown in DMEM and DMEM-F12K media, subsequently maintained as pellet cultures in the presence of TGF-β1 for 21 days. (A-F) Passage 1; (G-L) Passage 5. DMEM cultures from both passages exhibit larger pellets with a more abundant, alcian blue-stained sulfated proteoglycan matrix as compared with DMEM-F12K cultures. Cells also have a more organized appearance in DMEM cultures. Successively higher magnifications are shown: Bar = 300 μm (A, D, G, J); 80 μm (B, E, H, K); or 40 μm (C, F, I, L).

increasing passage number, indicating a general progression of cells into a state of replicative senescence [14]. Since serially subcultured DMEM cultured cells maintained under control conditions, i.e., without osteogenic, adipogenic, or chondrogenic stimuli, did not express markers of terminal differentiation after long-term culture and also retained the ability to differentiate along mesenchymal lineages upon subsequent stimulation, it is reasonable to conclude that these cells did not senesce by entering into a state of terminal differentiation [14, 32, 33]. This transition to a cell population with diminished replicative capacity occurred much more rapidly for DMEM-F12K cultures.

Primary cell cultures followed normal, predictable growth curves with lag, exponential, and plateau growth phases; however, the characteristics of each phase varied between DMEM and DMEM-F12K cell populations through long-term culture. DMEM cultures consistently exhibited a shorter lag phase as well as a steeper exponential growth phase, resulting in a higher cellular density before proliferation was inhibited by cell-cell contact. These cultures were able to maintain a linear increase in population doubling through an average of 12 passages before the rate of population growth began to decline. Passaged cultures proceeded through the same stages; however, the rate of growth in the log phase and the final number of cells after a fixed period in culture gradually diminished as a function of continued passaging. This decrease in the rate of growth, as well as the total number of cells as a function of increasing passage number, could be attributed to a small population of cells in each passage developing the broad, flattened morphology characteristic of cells entering G_0, thus resulting in a smaller number of actively dividing cells remaining in the population [14, 30, 34]. Therefore, loss of doubling potential was accelerated in the DMEM-F12K cultures by the onset of cellular senescence.

Additionally, the two culture media were found to differentially affect expression of the osteoblastic, adipogenic, and chondrogenic phenotypes. Besides being more potent in stimulating cell growth, culturing in DMEM elicited a stronger differentiation-inducing effect as determined by RT-PCR mRNA phenotyping and histology. Serially passaged cells grown in DMEM and treated with OS consistently expressed several bone-specific markers, such as ALP, BSP, Col 1, and OC, as well as elevated ALPase activity, thus confirming the retention of osteogenic potential as a function of passage. The loss of osteogenic differentiation potential of DMEM cell cultures was concurrent with the onset of cellular replicative senescence, which occurred between passages 10 and 12. In contrast, cells serially cultured in DMEM-F12K medium showed diminished osteogenic potential as early as passage 5 upon OS treatment. This was indicated by a reduced level of ALPase activity, as well as by lower mRNA levels of OC and ALP. Since DMEM and DMEM-F12K cultures in the absence of OS treatment maintained low basal levels of ALPase activity and failed to express any osteogenesis-, adipogenesis-, and chondrogenesis-specific genes besides limited levels of Col I, the cells are likely to have become senescent during extensive passage and lost the ability to become osteogenic upon treatment, thereby failing to make a lineage commitment when induced. This early onset of senescence in DMEM-F12K cultures may also have prevented them from responding to the mitogenic effects of the osteoinductive medium, thereby contributing to the lower levels of OS-specific mRNA and ALPase activity, as compared with the highly OS-responsive DMEM cultures.

Trabecular bone-derived cells cultured in DMEM were also capable of chondrogenic differentiation upon serial subculture up to an average of passage 10 before their ability to produce a proteoglycan-rich extracellular matrix began to diminish. The increased pellet culture size, extracellular matrix production, and morphology and orientation of cells derived from DMEM cultures compared with those derived from DMEM-F12K medium cultures can be attributed to the presence of a higher percentage of multipotent cells that are able to respond to chondrogenic induction upon serial subculture, similar to osteogenically and adipogenically induced DMEM cell cultures. Thus, the onset of senescence in DMEM and DMEM-F12K medium cultures, which began with passage 10 and 5 cultures, respectively, can be directly correlated to the loss of multipotentiality, the onset of which was induced more rapidly in the latter cultures.

In conclusion, we have identified the presence of a $CD73^+$, STRO-1^+, $CD105^+$, $CD34^-$, $CD45^-$, $CD144^-$ cell population resident within collagenase-treated bone fragments, which upon migration established a homogenous population of cells with stem cell-like capabilities. We have also introduced a system of culturing trabecular bone-derived MPCs that best supports and maintains their optimal differentiation potential during long-term culture expansion. Similar to human bone marrow-derived MPCs, these cells, when cultured as described, have the characteristics of mesenchymal progenitors, displaying a stable undifferentiated phenotype as well as the ability to proliferate extensively while retaining the potential to differentiate exclusively along the osteogenic, adipogenic, and chondrogenic lineages, even when maintained in long-term in vitro culture [17, 35-37]. It is also noteworthy that several other connective tissues, including adipose and striated muscle [38-40], harbor MPCs with multilineage differentiation potential. It would be of interest to assess the developmental relationship of MPCs obtained from these different sources. Studies using such an in vitro model should provide insights into the molecular and cellular events that occur during lineage-specific mesenchymal differentiation and demonstrate their potential application for the repair and regeneration of damaged or diseased tissues.

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